Responses of soil microorganisms to elevated CO_2 in experiment sites of *Pinus sylvestriformis* and *Pinus koraiensis*

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Abstract: Responses of soil microbial activities to elevated CO_2 in experiment sites of *Pinus sylvestriformis* and *Pinus koraiensis* seedlings were studied in summer in 2003. The results indicated the number of bacteria decreased significantly ($p < 0.05$) under elevated CO_2 for *Pinus sylvestriformis* and *Pinus koraiensis*. Amylase and invertase activities in soil increased for *Pinus sylvestriformis* and decreased for *Pinus koraiensis* with CO_2 enrichment compared with those at ambient (350 $\mu mol \cdot mol^{-1}$). The size of microbial biomass C also decreased significantly at 700 $\mu mol \cdot mol^{-1}$ CO_2. Bacterial community structure had some evident changes under elevated CO_2 by DGGE (Denaturing Gradient Gel Electrophoresis) analysis of bacterial 16S rDNA gene fragments amplified by PCR from DNA extracted directly from soil. The results suggested that responses of soil microorganisms to elevated CO_2 would be related to plant species exposed to elevated CO_2.

Keywords: Bacterial community; Bacterial numbers; Elevated CO_2; Soil enzyme activity.

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Introduction

Most of studies about global changes mainly focused on the response of forest ecosystem to elevated atmospheric CO_2. Eelevated CO_2 often caused larger increases in dry weight (DW) and length of plant roots and faster growth of plant root, which possibly led to the increase of penetration of the soil profile (Norby *et al.* 1994; Piedad *et al.* 2002; Rogers *et al.* 1996). Additionally, the rate of root turnover (Pregitzer *et al.* 1995), the composition, and the chemistry structure of root exudation also may make changes with CO_2 enrichment. As a result, the amount and the quality of organic substances from plant root into the soil might make some change. Microbial activity in soil might be influenced by the above alterations under elevated CO_2. Biomass allocation of plant (Norby *et al.* 1994; Rogers *et al.* 1996; Pregitzer *et al.* 1995) and the quantity and quality of rhizodeposition (Cardon *et al.* 1996; Paterson *et al.* 1996; Hodge *et al.* 1998) maybe made change under elevated CO_2, which may influence the composition of community structure and the number of soil microorganisms (Piedad *et al.* 2002; Hu *et al.* 1999). The composition of community structure and the number of soil microorganisms mainly depend on plant-derived carbon and are often associated with specific plant species. So far, most of studies on responses of soil microorganisms to elevated CO_2 have been devoted to microbial biomass; however inconsistent results are reported (Zak *et al.* 2000b; Diza *et al.* 1993; Allen *et al.* 2000; Williams *et al.* 2000; Zak *et al.* 2000a).

The aim of this study was to investigate the effects of elevated CO_2 on soil microorganisms in experiment sites of *Pinus sylvestriformis* and *Pinus koraiensis*. The results will provide some basic data for the study of global change.

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Materials and methods

Study site and experimental design

The study site is located in the Changbai Mountain of China, with an altitude of 740 m (128°28′ E, 42°24′N). Average annual precipitation is about 450–550 mm and average annual air temperature is 3.3 ℃. Soil is classified as dark brown soil derived from volcano ashes. Soil parameters in detail are as follows:

Chemical properties	The content
N	0.34 ± 0.03
C	5.29 ± 0.52
P g kg^{-1}	0.19 ± 0.03
K g kg^{-1}	.0.67 ± 0.22

The experiment of CO_2 enrichment was performed at the end of April of 1998 in the open top chambers (OTCs), which consists of aluminium frames of 1.2 m in length, 0.9 m in width and height, and clear glass covers. The seeds of *P. koraiensis* and *P. sylvestriformis* were sown in April, 1998. The samples were treated in ambient CO_2 (350 $\mu mol \cdot mol^{-1}$), ambient CO_2 (350 $\mu mol \cdot mol^{-1}$) chambers, 500 $\mu mol \cdot mol^{-1}$ CO_2 chambers, and 700 $\mu mol \cdot mol^{-1}$ CO_2 chambers, respectively. The experiment was conducted during the growing season (from May to September). The soil type is dark brown soil derived from volcanic.

Soil sampling and storage

Soil samples were collected from the top 10 cm of the horizon in the middle of July in 2003. One part of the soil samples stored at – 20 ℃ were used to analyze bacterial community structure, and another part of the soil stored at 4 ℃ were used to analyze the activities of enzymes and estimate the microbial biomass C. The storing time of sample did not exceed two weeks.

The number of bacteria and the microbial activity

The number of bacteria was measured by CFU (colony -forming units). Microbial biomass C was determined by chlo-

roform fumigation-extraction methods and was titrated by 0.05 $mol \cdot L^{-1}$ $FeSO_4$. The result was calculated from the following formula: the size of soil microbial biomass C ($mg \cdot kg^{-1}$) = 2.64 Ec, where Ec = [(C extracted from fumigated soil) minus (C extracted from non - fumigated soil)] (Lin *et al.* 1999). Amylase activity was determined by 3, 5-dinitrosalicy lic acid colorimetry, and invertase activity was titrated by using 0.05 $mol \cdot L^{-1}$ sodium hyposulfite (Hoffmann 1968).

DNA extraction and PCR amplification

DNA of soil microorganism was extracted by proteinase K based on SDS method (Zhou *et al.* 1996).

PCR amplification was performed with the primers of 341f-GC (5' -CGCCCGCCGCGCCCCGCGCCCGTCCCGCCGCCCCCGC CCGCCTACGGGAGGCAGCAG - 3') and 907r (5' - CCGTCAATTMTTTGAGTTT-3') amplifying variable V8 region of 16S rDNA. PCR mixture (50 μL) contains 10 ×*Ex Taq* buffer (Mg^{2+} plus), dNTP Mixture (2.5 $mmol \cdot L^{-1}$), *TaKaRa Ex Taq* (5 $U \cdot \mu L^{-1}$), template DNA (10 ng), primer 341f-GC and 907r (30 pmol, respectively). A touchdown thermocycling program was used for PCR. Template DNA was denatured for 5 min at 95℃. The initial annealing temperature was 65°C, and this was decreased 1 °C every cycle for 20 cycles; finally, 15 cycles were performed at 45°C. The extension for each cycle was carried out at 72°C for 3 min, while the final extension was at 72°C for 10 min (Ercolini, *et al.*, 2003). PCR products were routinely detected in 1% (w/v) agarose gels (Zhou *et al.* 1996).

DGGE (Denaturing Gradient Gel Electrophoresis) analysis

PCR products were analyzed by DGGE using a Bio-Rad Dcode apparatus. And DGGE was performed by using 6% (w/v) acrylamide gels (ratio of acrylamide to bisacrylamide, 37.5:1) containing a 30% to 70% urea-formamide denaturing gradient (100% corresponded to 7 $mol \cdot L^{-1}$ urea and 40% [wt vol^{-1})]] formamide). Approximately 180-ng PCR product was loaded per sample in final volume of 60 μL. The gels were electrophoresed at 60℃ at 200 V for 6 h, and then stained with silver stain (Chai *et al.* 2003).

Results

Bacterial numbers and microbial biomass C

The number of bacteria in soil decreased significantly ($p < 0.05$) at elevated CO_2 for *P. sylvestriformis* and *P. koraiensis* compared with at ambient CO_2 and ambient CO_2 chamber (Table 1). The difference of bacterial number between ambient CO_2 and ambient CO_2 chamber was insignificant for *P. koraiensis*, however, the difference was significant ($p < 0.05$) for *P. sylvestriformis*. The result indicated that the greenhouse effect on bacterial numbers in OTCs was not neglected for *P. sylvestriformis*. Elevated CO_2 resulted in the decrease of the number of bacteria.

Soil microbial C, as a sensitive factor for environment changes, is a pool in soil nutrient cycling and an importantly available nutrient source for plant growth. The size of microbial biomass C decreased significantly ($p < 0.01$) at 700 $\mu mol \cdot mol^{-1}$ CO_2 compared with that at ambient, and was 93.34 $mg \cdot kg^{-1}$ and 93.30 $mg \cdot kg^{-1}$ for *P. sylvestriformis* and *P. koraiensis*, respectively (Table 2). The size of microbial biomass C at 500 $\mu mol \cdot mol^{-1}$ CO_2 had insignificant changes compared with that at ambient and ambient chamber. And the differences in the size of microbial biomass C between ambient and ambient chamber were also insignificant. This result indicated that the effect of elevated CO_2 on microbial biomass C was more significant at 700 $\mu mol \cdot mol^{-1}$ than at 500 $\mu mol \cdot mol^{-1}$.

Table 1. Effects of elevated CO_2 on soil microflora in seedlings experiment sites of *P. syvestriformis* and *P. koraiensis* in summer in 2003 (n=3)

CO_2 Treatments ($\mu mol \cdot mol^{-1}$)	Bacterial number ($cell \cdot g^{-1}$ soil)	
	Pinus sylvestriformis	*Pinus koraiensis*
700	$5.75 \pm 0.30 (10^7)$	$8.01 \pm 0.45 (10^5)$
500	$7.49 \pm 0.15 (10^7)$	$8.19 \pm 0.51 (10^5)$
Ambient CO_2 chamber	$93.40 \pm 0.73 (10^7)$	$53.80 \pm 0.15 (10^5)$
Ambient CO_2	$46.70 \pm 0.70 (10^7)$	$53.70 \pm 0.33 (10^5)$

Table 2. Response of soil microbial biomass C to elevated CO_2 in seedlings experiment sites of *P. sylvestriformis* and *P. koraiensis* in summer in 2003 (n=3)

CO_2 Treatments ($\mu mol \cdot mol^{-1}$)	*Microbial biomass C* ($mg \cdot kg^{-1}$)	
	Pinus sylvestriformis	*Pinus koraiensis*
700	93.34 ± 0.17	93.30 ± 1.11
500	206.11 ± 0.13	105.29 ± 0.93
Ambient CO_2 chamber	198.77 ± 0.36	106.10 ± 0.94
Ambient CO_2	213.45 ± 0.14	105.91 ± 1.32

Enzyme activity in soil

Responses of amylase and invertase activities to elevated CO_2 were studied in summer of 2003 and the results were shown in Fig.1. For *Pinus sylvestriformis*, amylase activity in soil increased by 28.0% ($p < 0.05$) at 700 $\mu mol \cdot mol^{-1}$ CO_2 and change a little at 500 $\mu mol \cdot mol^{-1}$ CO_2 compared with that at ambient CO_2, and invertase activity increased significantly under elevated CO_2 compared with that at ambient CO_2 ($p < 0.01$). For *P. koraiensis*, amylase activity in soil decreased by 8.5% at 700 $\mu mol \cdot mol^{-1}$ CO_2 and 26.9% ($p < 0.05$) at 500 $\mu mol \cdot mol^{-1}$ CO_2 compared with at ambient CO_2, and invertase activity decreased by 9.4% at 700 $\mu mol \cdot mol^{-1}$ CO_2 and 36.6% ($p < 0.01$) at 500 $\mu mol \cdot mol^{-1}$ CO_2 compared with that at ambient CO_2. There was insignificant difference in amylase and invertase activities between ambient CO_2 and ambient CO_2 chamber. Above results suggested that the different trends of amylase and invertase activities in soil in experiment sites of *P. koraiensis* and *P. sylvestriformis* seedlings under elevated CO_2 were associated with tree species, and the difference of different species in soil enzyme activities might be due to their different physiology responses to elevated CO_2.

Bacterial community structure

Bacterial community structure in soil from the experiment sites of *P. sylvestriformis* (A) and *P. koraiensis* (B) under different CO_2 concentrations were investigated in the summer of 2003 (Fig.2). The patterns showed bands of a range of intensities indicating proportional variations in community components. And there was some heterogeneity in bacterial community structure between elevated CO_2 and ambient in two experiment sites. Bacterial community structures had a change under elevated CO_2 compared with at ambient CO_2 although the dominant bacterial population had no changes from the Fig.2. And the profiles of DGGE showed that some species newly appeared or the number of indigenous bacterial species was enriched within the bacterial community structure exposed to elevated CO_2 in the experiment site of *P. sylvestriformis*. However, some populations disappeared or the number of indigenous bacterial species was weak-

ened with CO_2 enrichment for *P. koraiensis*. This result indicatedthat bacterial community structure in soil was affected by elevated CO_2. The results of DGGE profiles also suggested that the responses of bacterial community structure in soil to elevated CO_2 were different for two trees species (Hu *et al.* 1999).

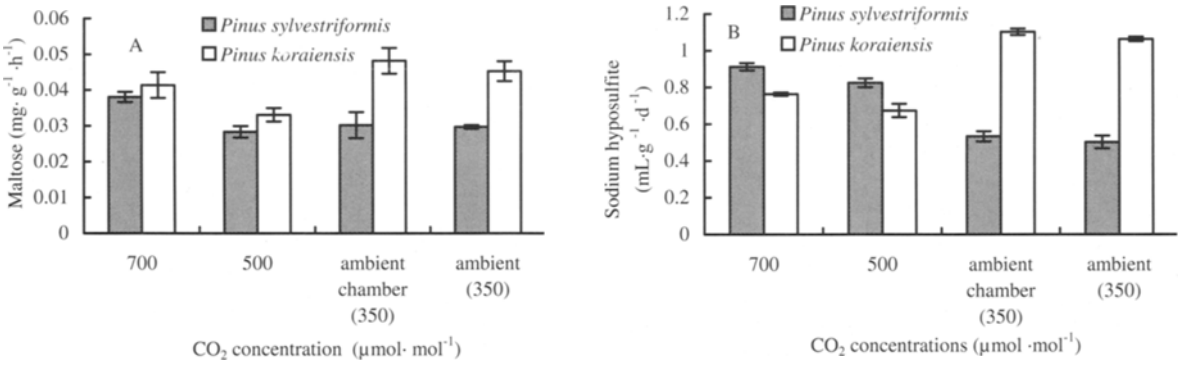

Fig.1 Soil enzyme activities under different CO_2 concentrations in two species of trees experiment sites in summer (A: Amylase, B: Invertase)

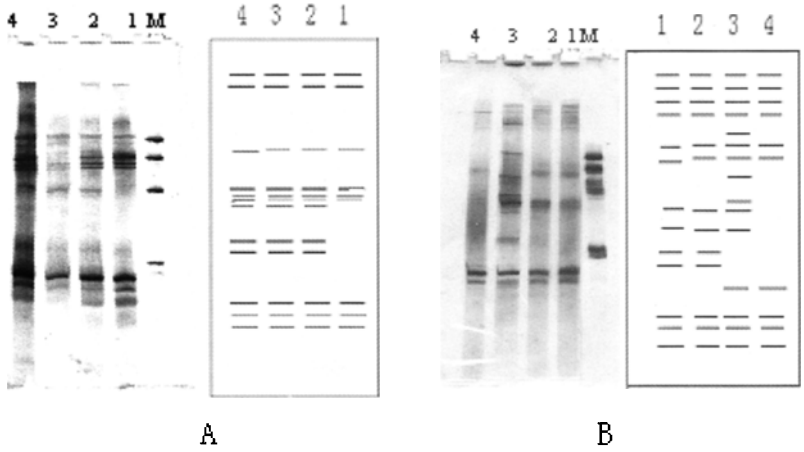

Fig.2 DGGE profiles of 16S rDNA genes fragments amplifiled from DNA extracted from the soil in *Pinus sylvestriformis* (A) and *Pinus koraiensis* (B) experimsnt sites under different CO_2 concentrations in summer.

Notes: Lanes 1-4 were at 700 μmol · mol^{-1}, 500 μmol · mol^{-1}, ambient chamber, and ambient, respectively.

Discussion

Soil microorganisms play a central role in major ecosystem processes such as the transformation of energy and nutrients and microbial communities, and they are endemic to their particular environment. Most of studies indicated that dry weight of root, root growth, below-ground carbon allocation, rhizodeposition, and the rate of root turnover always increased for most of plants exposed to elevated CO_2; And the quantity (Rogers *et al.* 1996; Pregitzer *et al.* 1995), the composition, and the chemistry structure of root exudation might alter by effects of CO_2 enrichment on plant physiology. So the amount and the quality of organic substances into the soil and soil structure might make some changes. For example, Niklaus *et al.* (2003) found some changes in soil structure after studying nutrient-poor grassland exposed to CO_2 enrichment for six years.

To date, only a few studies addressed structural changes within the microbial communities with CO_2 enrichment (Zak *et al.* 2000a; Wiemken *et al.* 2001; Ringelberg *et al.* 1997). Bruce *et al.* (2000) also could not find changes in DGGE profiles of bacterial communities from model terrestrial ecosystems exposed to elevated CO_2, but Montealegre *et al.* (2000) found changes in PLFA (Phospholipid Fatty Acid) profiles in bulk soil of *Trifolium repens* exposed to elevated CO_2, which was in line with our result. Our result suggested that the changes of bacterial community structure was related to plant species exposed to elevated CO_2, which also proved the results obtained by Grayston *et al.* (1998).

The changes of the number of soil bacterial, microbial biomass C, enzyme activity in soil, and bacterial community structure in this study suggested that soil microbial activities can be affected by elevated CO_2. The decrease of the number of soil bacteria (depending on decomposed root, exudation, and litters and so on) would probably be associated with the responses of physiology of *Pinus* to elevated CO_2 (Zhou *et al.* 2002). And root growth, below-ground carbon allocation, and rhizodeposition and so on would be slower under elevated CO_2 compared with at ambient. All of these probably resulted in the decrease of bacterial numbers under elevated CO_2 compared with under ambient.

The size of microbial biomass C decreased significantly at 700 μmol · mol^{-1} CO_2, which disagreed with Niklaus's studies (1998), who found microbial biomass carbon was not influenced by elevated CO_2. The activities of invertase and amylase for two trees species can be affected by elevated CO_2. Many studies indicated that available-C to microorganism increased (Ross *et al.* 1996; Hungate *et al.* 1997b; Ebersberger *et al.* 2003) under elevated CO_2. The increase of soil enzyme activities for *P. sylvestriformis*

under elevated CO_2 could also be associated with the increase of available C to microorganism and N- mineralisation. The different trends of soil enzyme activities between *P. koraiensis* and *P. sylvestriformis* with CO_2 enrichment were seemly associated with different physiology responses of different tree species to elevated CO_2. Additionally, the DGGE profiles from DNA extracted from soil in the experiment site of *P. sylvestriformis* also indicated that bacterial community structure was influenced by elevated CO_2, which might cause changes of part of soil amylase and invertase derived from microorganisms. This result indirectly showed that enzyme activities in soil were also affected by the change of bacterial community structure under elevated CO_2.

The result of DGGE profiles indicated some populations newly appeared or the number of indigenous bacterial species was enriched within the bacterial community structure exposed to the elevated CO_2 in the experiment site of *P. sylvestriformis*. However, some populations disappeared or the number of indigenous bacterial species was weakened with CO_2 enrichment in experiment site of *P. koraiensis*.

Given that the concentration of CO_2 in soil is 10-50 times higher than that in the atmosphere (Lmaborg *et al.* 1998), a direct response to elevated atmospheric CO_2 in terms of bacterial numbers, microbial biomass C, amylase and invertase activities, and bacterial community structure was unlikely (Bruce *et al.* 2000). However, responses of soil microbia to elevated CO_2 in our study occurred. So we believed they occurred by mainly indirect means of effects of CO_2 enrichment on plant physiology (e.g. root growth, rhizodeposition, rate of root turnover, and so on). Of course, greenhouse effects from OTCs might also be neglected in the study.

Conclusion

Microbial community structure and its activities in soil were affected by elevated CO_2 in experiment sites of *P. sylvestriformis* and *P. koraiensis*. The results indicated bacterial numbers in soil decreased significantly ($p < 0.05$) for *P. sylvestriformis* and *P. koraiensis* with CO_2 enrichment. Amylase and invertase activities in soil increased for *P. sylvestriformis* and decreased for *P. koraiensis* under elevated CO_2 compared with at ambient CO_2. The size of microbial biomass C also decreased significantly at 700 $\mu mol \cdot mol^{-1}$ CO_2. And the bacterial community was affected distinctly by elevated CO_2 through DGGE profiles.

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